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Translational efficiency of the *Escherichia coli* adenylate cyclase gene: Mutating the UUG initiation codon to GUG or AUG results in increased gene expression

(*cya:galK* fusions/oligonucleotide-directed mutagenesis)

PRASAD REDDY*, ALAN PETERKOFSKY*, AND KEITH MCKENNEY†

*Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute and †Laboratory of Molecular Genetics, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD 20205

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ABSTRACT **Roy *et al.* [Roy, A., Haziza, C. & Danchin, A. (1983) *EMBO J.* 2, 791–797] established that translation of *Escherichia coli* adenylate cyclase initiates at a UUG codon, and they suggested this might decrease the efficiency of translation. We investigated the effect of varying the initiation codon on the expression of the adenylate cyclase (*cya*) gene. Using oligonucleotide-directed mutagenesis, we changed the UUG initiation codon to GUG and the more common initiator AUG and assayed for *cya* gene expression in a number of ways. First, the GUG initiation codon, in place of UUG, doubled *cya* expression when *cya* was expressed from the dual *cya* P_1/P_2 promoters. The corresponding AUG codon construct was nonviable. Second, when the *cya* gene was placed under the transcriptional control of the thermoinducible phage λ P_L promoter, the relative amounts of *cya* gene product were 1:2:6 for the UUG, GUG, and AUG initiation codons, respectively. Finally, the *cya* P_2 promoter, Shine–Dalgarno sequence, and the DNA corresponding to the first 86 codons of *cya* were fused to DNA encoding the *E. coli* galactokinase gene beginning at the second codon. The relative amounts of the fusion polypeptides, which had galactokinase activity, were 1:2:3 for the UUG, GUG, and AUG initiation codons, respectively. These results demonstrate that the *cya* UUG initiation codon limits *cya* expression at the level of translation.**

Translation initiation requires an initiation codon and a properly spaced sequence upstream of the initiation codon that has base pairing potential with the 3′ end of the 16S ribosomal RNA (the Shine and Dalgarno or SD sequence) (1, 2). AUG, GUG, and UUG triplets have been shown to stabilize the binding of initiator fMet-tRNA to ribosomes *in vitro*, suggesting that these three codons may function *in vivo* as initiation codons (3). Comparison of protein sequence data with the corresponding nucleic acid sequence data for more than 100 genes has shown that, *in vivo*, all eukaryotic and nearly all prokaryotic genes use the translation initiation codon AUG. However, there are 9 examples of GUG and 3 examples of UUG functioning as initiation codons in *Escherichia coli* (2, 4). The gene for *E. coli* adenylate cyclase (*cya*), the enzyme that synthesizes the important cellular regulator cAMP, is one of the genes that uses the unusual UUG initiation codon (4–6).

We have investigated the effect of this unusual initiation codon on *cya* expression by changing the DNA sequence coding for the UUG initiation codon to ATG and GTG, using oligonucleotide-directed mutagenesis. A comparison of the activities associated with the three codons was made in three different environments: (*i*) in the normal *cya* environment, with the *cya* gene expressed from the dual *cya* P_1/P_2 promoters, (*ii*) in a transcription fusion with the *cya* gene under the transcriptional control of the phage λ P_L promoter, and (*iii*) in a gene fusion with the *cya* gene fused to the *E. coli* galactokinase (*galK*) gene to generate a fusion protein with galactokinase activity. In each of these three environments, it was observed that the UUG initiation codon had the lowest efficiency of translation initiation and the AUG initiation codon had the highest efficiency, while the GUG initiation codon was intermediate. This is consistent with the finding that the cellular concentration of adenylate cyclase is very low (ref. 7 and unpublished work).

MATERIALS AND METHODS

***E. coli* Strains.** C600(r^- m^+ λ lysogen) and MZ1(*galK*$^-$, λ lysogen carrying *c*I857ts) were kindly provided by D. Court of the National Cancer Institute. GM33(*dam*$^-$) was obtained from H. Nash of the National Institute of Mental Health. Other strains used were C600(*galK*$^-$) and JM101(Δ*lac*) (8).

Plasmids. pDIA100 (4, 9) encoding the *cya* promoters, SD sequence, and structural gene was kindly provided by A. Danchin of the Pasteur Institute (Paris, France). pKC30 (10), which contains the λ P_L promoter, was a gift from Y. Ho of Smith Kline & French Laboratories (Philadelphia, PA). pKgalS, containing the entire *E. coli gal* operon (11), was obtained from D. Schumperli of the University of Zurich. pK0500 contains the *E. coli galK* gene with the polylinker derived from M13mp11 (unpublished data). M13mp8 (12) was obtained from S. Satchwell of the Medical Research Council (Cambridge, England).

Other Materials. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and the large fragment of DNA polymerase I were from New England Biolabs or International Biotechnologies (New Haven, CT). The deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate was purchased from New England Nuclear. The synthetic oligonucleotides 5′-AGAGGTACA$\begin{matrix}T\\C\end{matrix}$GACGTATCG-3′ with a T/C degeneracy at position 10 were purchased from Oligonucleotide Chemistry Synthesis Laboratories (Denton, TX). All other chemicals were of analytical grade.

Plasmid DNA. Strains C600(λcI^+) or GM33 carrying plasmids were grown at 37°C to stationary phase in Luria–Bertani (LB) medium (13) containing ampicillin (25 μg/ml). Plasmid DNA was isolated by lysozyme/alkaline sodium dodecyl sulfate extraction as described by Birnboim and Doly (14) and purified by centrifugation to equilibrium in cesium chloride/

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Abbreviations: SD, Shine–Dalgarno; RF, replicative form; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; bp, base pair(s).

ethidium bromide gradients (15). Minipreparations of plasmid DNA were isolated by the lysozyme/alkaline sodium dodecyl sulfate method (14) and treated with RNase.

M13 Phage DNA. JM101 infected with M13 phage was grown in LB medium with vigorous aeration for 5 hr at 37°C. Single-stranded DNA templates were isolated from culture supernatants as described (16). The replicative form (RF) of M13 DNA was isolated as follows: JM101 grown at 37°C to early logarithmic phase in 2× YT broth (13) were infected with M13 phage at a multiplicity of infection of 30 and growth was continued for 1 hr. Chloramphenicol amplification and isolation of RF DNA were performed as described (8). RF DNA was purified by equilibrium centrifugation in cesium chloride/ethidium bromide gradients.

Restriction Enzyme Digestion, Ligation, and Transformation. DNA digestion and ligation reactions were performed as described by Maniatis *et al.* (15). Competent cells for transformation were made by the Hanahan method (17). Routinely, C600(λcI^+) was used to transform with the ligation mixtures and to isolate hybrid plasmids. When MZ1 was transformed with P_L-containing plasmids, heat shock was done at 30°C and plates were incubated at 30°C. Competent cells of JM101 were prepared by treating with $CaCl_2$ (8).

Isolation of DNA Fragments. DNA fragments were separated by agarose gel electrophoresis, and gel slices were extracted with phenol and the DNA in the aqueous phase was precipitated with ethanol (15). DNA fragments were purified on NACS-Prepac columns (Bethesda Research Laboratories) according to the manufacturer's specifications.

Oligonucleotide-Directed Mutagenesis. The synthetic oligonucleotides were annealed with an M13 DNA template containing a fragment of DNA [524 base pairs (bp)] encoding the region around the initiation codon of the *cya* gene. The second strand was synthesized in the presence of deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate, dCTP, dGTP, dTTP, and the large fragment of DNA polymerase I, and T4 DNA ligase for 15 min at 23°C. Synthesis was continued at 15°C for 16 hr after unlabeled dATP had been added as described by Winter *et al.* (18). Closed circular (cc) DNA was isolated by centrifugation on an alkaline sucrose gradient. Competent JM101 cells were transfected with purified cc DNA and single-stranded DNA templates were isolated for characterization (16).

Growth and Heat Induction of Bacteria. C600(λcI^+) and C600($galK^-$) carrying recombinant plasmids were grown at 37°C in Vogel and Bonner medium E (19) containing 0.8% Difco nutrient broth, 0.5% glucose, and ampicillin (25 μg/ml). When MZ1 carrying pKC30 derivatives was used for measuring expression of adenylate cyclase or galactokinase, cells were grown at 30°C to early logarithmic phase (OD_{650} of 0.3–0.4), an equal volume of the medium warmed to 65°C was added, and growth was continued at 42°C for 2 hr (20).

Enzyme Assays. Adenylate cyclase activity in French press extracts was determined as described (21). Briefly, 30 ml of uninduced cells or 10 ml of induced cells was harvested, and the cells were washed with 25 mM *N,N*-bis(2-hydroxyethyl)-glycine (Bicine), pH 8.5, and suspended in 1 ml of 25 mM Bicine. Cells were passed twice through an Aminco French pressure cell at 11,000 pounds/inch^2 (76 MPa). Galactokinase activity in suitably diluted extracts was measured as described by McKenney *et al.* (22).

Protein was estimated by the Lowry method (23).

RESULTS

Changing the DNA Sequence Coding for the UUG Initiation Codon of *cya* to ATG and GTG. The initiation codon for adenylate cyclase was shown to be UUG by comparing the protein sequence of a *cya:lacZ* fusion protein with the *cya* DNA sequence (5). To study the effect of the initiation codon on the translational efficiency of *cya* mRNA, we changed the naturally occurring UUG triplet to AUG or GUG by oligonucleotide-directed mutagenesis. pDIA100 DNA (9) was the source of the *cya* gene in the experiments described here. The 524-bp *Bam*HI fragment of pDIA100, which contains the *cya* P_2 promoter, the SD sequence, and the first 86 codons of *cya* (Fig. 1) was inserted into the *Bam*HI site of M13mp8 (12). The orientation and nucleotide sequence of the fragment were determined by the chain termination method of Sanger *et al.* (24), using the primer LMB2 (25). Our sequence data confirmed the published DNA sequence of this fragment of *cya* (6). A mixture of two 19-base synthetic oligonucleotides (5′-AGAGGTACA$\begin{matrix}T\\C\end{matrix}$GACGTATCG-3′) identical except for a T/C degeneracy at position 10 was used to change the TTG to ATG and GTG as described in *Materials and Methods*. The T-to-A and the T-to-G mutations were identified by DNA sequencing using the primer LMB2. Except for the first base of the initiation codon, the rest of the sequence was wild type. A total of 40 independent clones were sequenced, of which 35% had the T-to-A change and 15% had the T-to-G change. One clone of each class (M13ATG and M13GTG) was selected for reconstructing the *cya* gene with mutations in the initiation codon.

Constructing the *cya* Gene with AUG and GUG Initiation Codons. To construct the *cya* gene with the AUG and GUG initiation codons, the *Bam*HI fragments of the M13ATG and M13GTG RF DNA were ligated into the *Bam*HI site of pPR3, a plasmid derived from pDIA100 by deleting the sequence between the two *Bam*HI sites of pDIA100 (Fig. 1). The *Bam*HI fragment has the potential to be inserted in either orientation in pPR3. In one orientation the *cya* gene is reconstructed with the *cya* gene expressed from the *cya* promoters. In the other orientation, the *cya* gene is disrupted and not functional. When the fragment *Bam*HI-GTG-*Bam*HI was ligated to pPR3 and used to transform *E. coli* C600(λcI^+), 50% of the recombinant plasmids examined had the fragment in the functional orientation and 50% had the fragment in the nonfunctional orientation as determined by restriction enzyme analysis and adenylate cyclase activity. One of the plasmids (pPR712) with the *Bam*HI-GTG-*Bam*HI fragment in the functional orientation expressed approximately twice as much adenylate cyclase activity as strain C600(λcI^+) transformed with pDIA100 containing the UUG initiation codon (data not shown). In contrast, when the insert fragment was

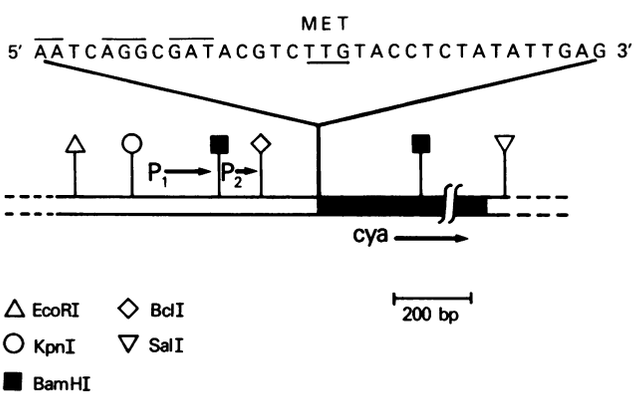

FIG. 1. Partial restriction map of the *cya* gene of *E. coli* in pDIA100. The restriction sites shown in this figure were adopted from Roy and Danchin (9). Relevant restriction sites used for subcloning are indicated by the symbols. The shaded area represents the structural gene and the arrows, the direction of transcription. The location of the promoters is represented by P_1 and P_2. The nucleotide sequence 3′ and 5′ to the initiation codon (underlined) is shown. The proposed SD sequence is overlined (4).

*Bam*HI-ATG-*Bam*HI, 39 of 40 recombinant plasmids examined had the fragment in the nonfunctional orientation. One of these plasmids (pPR16) was used as a source of the *Bcl* I-ATG-*Bam*HI fragment (see below). A fine restriction map generated with *Hae* III showed that the plasmid with the fragment in the functional orientation had a small deletion surrounding the *Bam*HI site in the *cya* coding sequence; tests for enzyme activity (21) showed that this plasmid did not code for active enzyme. This suggested that expression of the *cya* gene from the *cya* P_1 and P_2 promoters with an AUG initiation codon on a multicopy plasmid in *E. coli* C600 is lethal. Two solutions to this lethal *cya* expression were apparent. The first was to disrupt the *cya* coding region, thereby inactivating adenylate cyclase, and the second was to tightly control the transcription of the *cya* gene.

Construction of *cya:galK* Fusions. To construct the *cya* gene with AUG, GUG, or UUG initiation codons and disrupt the *cya* coding sequence, yet still quantitate the effect of the different initiation codons on the efficiency of translation initiation at *cya*, fusions of DNA fragments coding for the *cya* P_2 promoter, SD sequence, and the first 86 codons of *cya* with UUG, GUG, or AUG as initiators to the second codon of the *E. coli* galactokinase gene were made. These fusions code for hybrid *cya:galK* polypeptides devoid of adenylate cyclase activity but exhibiting galactokinase activity. This was accomplished by using plasmid pPR11, which has the *galK* gene with a polylinker at the second codon of *galK* and is devoid of transcription and translation initiation signals for *galK*. (Details of this construction will be described elsewhere.) The 524-bp *Bam*HI fragments from pDIA100 (TTG initiation sequence), pPR712 (GTG), and pPR16 (ATG) were inserted into the *Bam*HI site of plasmid pPR11 (Fig. 2). The inserts were oriented by restriction mapping and the *cya:galK* fusion peptides were screened for complementation in strain C600(*galK*$^-$) on MacConkey plates containing galactose (1%) and ampicillin (35 μg/ml). The resulting plasmids with the *Bam*HI fragment in the functional orientation were designated as pPR92(TTG), pPR72(GTG), and pPR52(ATG) (Fig. 2). The expression of the fusion proteins was quantitated by using the galactokinase assay. The relative amounts of galactokinase synthesized by cells harboring the three plasmids (TTG:GTG:ATG) were 1:2:3, as shown in Fig. 3.

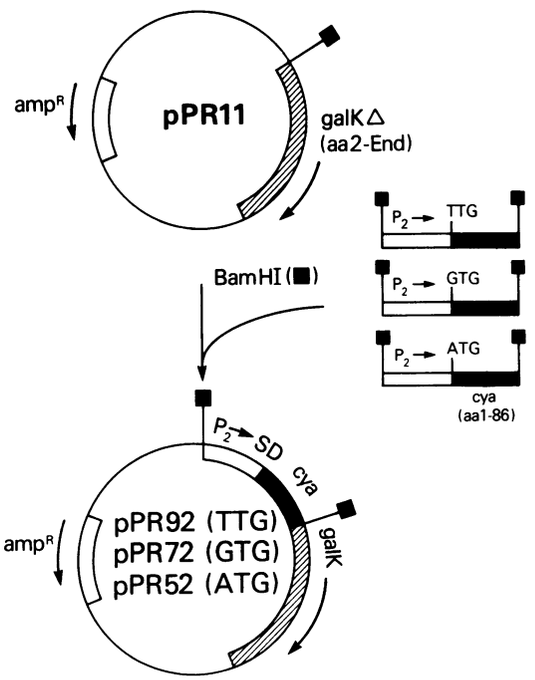

FIG. 2. Schematic representation of the construction of *cya:galK* fusion plasmids. pPR11 has part of the *galK* structural gene, without its own SD sequence and AUG initiation codon, coding for the second through the last amino acid (hatched region). *Bam*HI–*Bam*HI fragments derived from pDIA100, pPR712, and pPR16 carrying the *cya* P_2 promoter, the SD sequence, and part of the *cya* structural gene (shaded regions) initiating with UUG, GUG, or AUG were inserted into the *Bam*HI site of pPR11 to produce the *cya:galK* fusions under the control of the *cya* P_2 promoter. *amp*^{R}, Ampicillin-resistance gene.

Placing the *cya* Gene with Various Initiation Codons Under the Control of the λ P_L Promoter. Because we could not obtain a functional *cya* gene when transcription was from the *cya* P_1 and P_2 promoters with an AUG initiation codon, we constructed a functional *cya* gene with an AUG initiation codon (and, in addition, GUG and UUG initiation codons for comparing the efficiency of translation) under the control of the λ P_L promoter. In addition, to quantitate the transcription through *cya* from P_L, the *E. coli galK* gene was positioned distal to *cya* (Fig. 4). The *Eco*RI-*Sal* I fragment encoding the *cya* gene from pDIA100 (Fig. 1) was inserted between the *Hpa* I and *Sal* I sites of pKC30 (10) to make pPR5. A DNA fragment coding for the *galK* gene with the *galK* SD sequence and AUG initiation codon was isolated from pKgalS (11) as a *Pvu* II–*Hpa* I fragment and inserted into the *Nru* I site of pPR5 to make pPR621 (Fig. 4). Removal of the *cya* promoters, SD sequence, and first 86 codons of *cya* on pPR621 was accomplished by deleting the DNA sequence between the *Kpn* I and *Bam*HI sites. This resulting plasmid was designated pPR33 (Fig. 4).

To reconstruct functional *cya* genes with UUG, GUG, or AUG initiation codons, *Bcl* I-*Bam*HI fragments coding for the *cya* SD sequence and the first 86 codons were isolated from pDIA100, pPR712, and pPR16, respectively, and inserted into the *Bam*HI site of pPR33 (Fig. 4). The *Bcl* I–*Bam*HI fragment can insert in either a functional orientation, reconstructing the *cya* gene, or in a nonfunctional orientation, with a disrupted *cya* gene. Recombinant plasmids were isolated and characterized by restriction mapping and *cya* expression. The resulting plasmids with the *Bcl* I–*Bam*HI fragment in

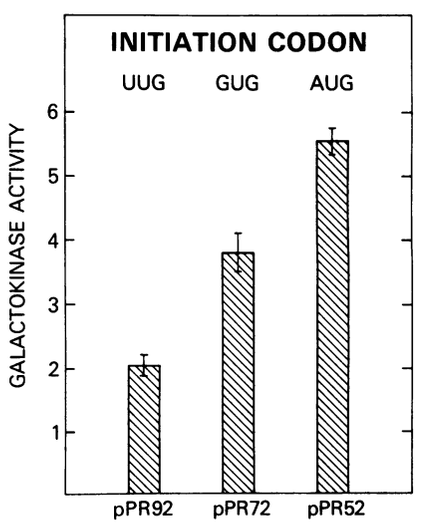

FIG. 3. Expression of galactokinase activity in *cya:galK* fusions under the control of the *cya* P_2 promoter. *E. coli* C600(*galK*$^-$) carrying the *cya:galK* plasmids pPR92, pPR72, and pPR52 with UUG, GUG, and AUG as initiation codons, respectively, were grown at 37°C to an OD_{650} of 0.5. Aliquots (10 ml) of cultures were centrifuged at 3000 × *g* for 10 min and washed with 25 mM Bicine, pH 8.5. Cells were suspended in 1 ml of Bicine and passed twice through a French pressure cell at 11,000 pounds/inch^2. Extracts were assayed for galactokinase activity at 30°C as described (22). Galactokinase activity is expressed as nmol of galactose phosphorylated per min per ml of cells at OD_{650} = 1.

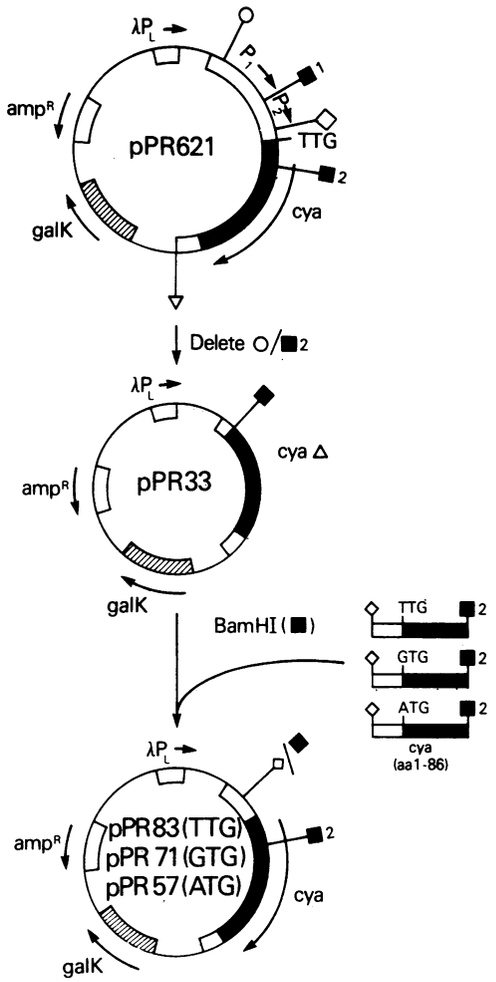

FIG. 4. Schematic representation of the construction of the *E. coli cya* gene with UUG, GUG, and AUG initiation codons under the control of the λ P_L promoter. pPR621, a derivative of pKC30, containing the *cya* promoters P_1 and P_2, the *cya* structural gene (shaded region), and the *galK* structural gene (hatched region) was constructed as described in *Results*. pPR33 was derived from pPR621 by deletion of the DNA fragment (*Kpn* I–*Bam*HI) encoding the *cya* promoters and first 86 codons of the structural gene. The *Bcl* I–*Bam*HI fragments derived from pDIA100, pPR712, and pPR16 were inserted into the *Bam*HI site of pPR33 to produce pPR83, pPR71, and pPR57 with the UUG, GUG, and AUG initiation codons, respectively. Symbols designating restriction sites are identical with those shown in Fig. 1.

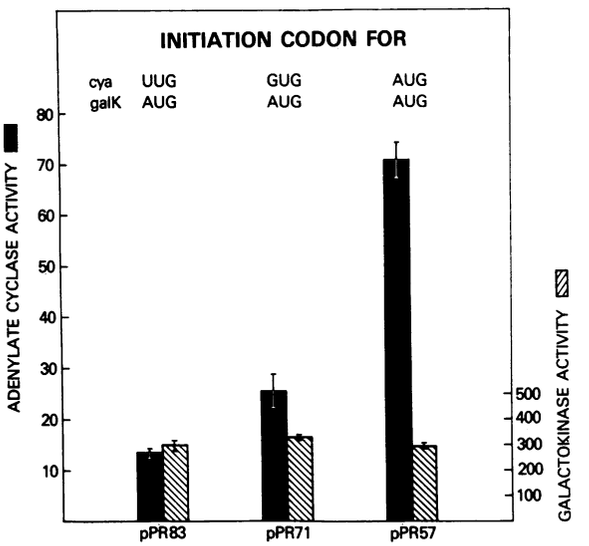

FIG. 5. Expression of adenylate cyclase and galactokinase activities under the control of the λ P_L promoter. *E. coli* MZ1 carrying plasmids pPR83, pPR71, and pPR57 with UUG, GUG, and AUG initiation codons for adenylate cyclase, respectively, were grown at 30°C to an OD_{650} of 0.3–0.4. After 30-ml samples had been removed for measuring adenylate cyclase activity in uninduced cells, an equal volume of fresh medium prewarmed to 65°C was added to the remainder of the culture and growth was continued for 2 hr at 42°C. Ten-milliliter aliquots of temperature-induced cells and 30-ml aliquots of uninduced cells were harvested at 3000 × *g* for 10 min and washed with 25 mM Bicine, pH 8.5. The cells were suspended in 1 ml of Bicine and passed twice through a French pressure cell at 11,000 pounds/inch^2. Extracts were assayed for adenylate cyclase activity at 30°C as described (21). Adenylate cyclase activity is expressed as nmol of cAMP formed per hr per mg of protein. The uninduced activity of adenylate cyclase in *E. coli* MZ1 was ≈1 nmol of cAMP formed per hr per mg of protein. Galactokinase activity was determined at 30°C (22) in the same extracts as used for adenylate cyclase measurements. Galactokinase activity is expressed as nmol of galactose phosphorylated per min per ml of cells at OD_{650} = 1.0. Galactokinase activity in extracts of the uninduced cells was 7 nmol of galactose phosphorylated per min per ml of cells at OD_{650} = 1.0.

the functional orientation are designated pPR83(TTG), pPR71(GTG), and pPR57(ATG) (Fig. 4). *E. coli* MZ1 was transformed with the three plasmids. Cultures of the transformants were heat induced and extracts were prepared for measurement of adenylate cyclase activity. As shown in Fig. 5, the relative amounts of adenylate cyclase activity were 1:2:6 for the constructs with the UUG, GUG, and AUG initiation codons.

Quantitation of P_L Transcription Through *cya*. The data of Figs. 3 and 5 indicate that the change of a single base pair in the DNA coding for the *cya* initiation codon increases *cya* expression. We considered and eliminated the possibility that this increased expression results from differential transcription in the plasmids with the three different initiation codons. We quantitated the transcription from the P_L promoter through *cya* by measuring the activity of galactokinase synthesized from a distal *galK* gene in plasmids pPR83(TTG), pPR71(GTG), and pPR57(ATG) (see Fig. 4). The amount of galactokinase synthesized by each plasmid is nearly identical (Fig. 5). These data indicate that the initiation codon-dependent variation in *cya* expression detailed in Figs. 3 and 5 must occur at the translation level.

DISCUSSION

The finding that the structural gene for *E. coli* adenylate cyclase is initiated by the unusual initiation codon UUG (4) prompted an investigation of the significance of the UUG initiation codon for the translational efficiency of the gene. Other examples of the occurrence of UUG initiation codons in *E. coli* are the ribosomal protein S20 gene (26) and the NADH dehydrogenase gene (27).

The work presented here constitutes a systematic study of the effect of changes of an initiation codon from UUG to AUG or GUG on the translational efficiency of a gene. The first base (T) of the DNA sequence coding for the initiation codon of *cya* was changed to A or G by using the technique of oligonucleotide-directed mutagenesis on a fragment containing the NH_2-terminal portion of the *cya* gene in an M13mp8 vector (12). To quantitate the effects of initiation codon changes on *cya* expression, the *cya* fragment was excised from the M13 replicative form DNA and reinserted into a plasmid (pPR3) containing the remaining *cya* sequence, thereby reconstructing an intact *cya* gene.

As described in *Results*, we were unable to isolate a plasmid containing the ATG initiation sequence under control of the *cya* P_1 and P_2 promoters in *E. coli* C600(λcI^+) but were successful in constructing such a clone with a GTG

initiation sequence and observed that this resulted in a doubling in the amount of the *cya* gene product. We suggest that, under these conditions, the ATG initiation sequence results in overexpression of adenylate cyclase with concomitant overproduction of cAMP to growth-inhibitory levels (28).

We quantitated the effect of changing UUG to GUG or AUG initiation codons in two ways. The first was a gene fusion experiment in which the first 86 codons of *cya* were fused to the second codon of the *E. coli galK* gene in phase to make a fusion gene under the control of the *cya* P_2 promoter. Since the fusion gene expressed a protein that had galactokinase but not adenylate cyclase activity, the accumulation of toxic levels of cAMP was avoided.

The second approach to quantitate the effect of changing the initiation codon from UUG to GUG or AUG was to replace the *cya* transcription elements with the controllable λ P_L promoter. In this environment, the P_L promoter is repressed at 32°C by the thermolabile λ *c*I857 repressor supplied in *trans* from a defective λ prophage on the *E. coli* chromosome in the host MZ1. We were therefore able to grow such cells at 32°C without accumulating lethal levels of cAMP. Shifting the temperature of a midlogarithmic-phase culture from 32°C to 42°C results in the inactivation of the *c*I857 repressor and the activation of the P_L promoter.

In both types of experiment, it was observed that the level of gene products was increased as a result of changing the initiation codon from UUG to GUG or AUG. In the case of the *cya:galK* fusions, where galactokinase activity was assayed, the activity ratio was UUG:GUG:AUG = 1:2:3 (Fig. 3). In the case of the *cya* gene expressed under the control of the P_L promoter, the activity ratio was UUG:GUG:AUG = 1:2:6 (Fig. 5). We examined the possibility that the 2-fold variation in the effects in these experiments was due to the difference in the temperatures used for culturing the cells (37°C in the case of the *cya:galK* fusions; 42°C in the case of the P_L promoter-controlled *cya*). The strains harboring the plasmids encoding the *cya:galK* fusions were grown at different temperatures and assayed for galactokinase activity. We expected to see a higher AUG-to-UUG ratio at higher culture temperature. To our surprise, we found (data not shown) that the UUG-to-AUG ratio for galactokinase activity in the *cya:galK* fusion protein was 1:5 at 30°C; 1:3 at 37°C, and 1:2 at 42°C. Therefore, it appears that the temperature at which cells are grown influences the relative translation efficiency for AUG and UUG initiation codons. The results of this experiment suggest that the UUG-to-AUG ratio in the construct under the control of the P_L promoter might be greater than 1:6 if the P_L promoter were activated at a temperature lower than 42°C. It is premature to predict whether the magnitude of the differences in translation efficiency between UUG and AUG initiation codons observed in this study (1:3 to 1:6) might be observed with other genes. It is noteworthy that *cya* uses an atypical SD sequence (see Fig. 1); this as well as other features of the *cya* gene may be important in determining the efficiency of translation.

Changes in adenylate cyclase activities associated with changing the initiation codon for *cya* might be the result of changes in either transcription or translation. We were able to distinguish between these two possibilities by measuring galactokinase activities in the constructs in which the *galK* gene, with its own SD sequence and AUG initiation codon but devoid of promoter, was placed downstream of the *cya* gene under the control of the λ P_L promoter (Fig. 4). The observation that, while there was a codon-dependent change in the level of adenylate cyclase activities, there was no change in the level of galactokinase activities established that there was no transcriptional effect due to the codon changes in the *cya* gene (Fig. 5.).

In this study, we have demonstrated that specific changes in the initiation codon of the *cya* gene result in changes in the efficiency of translation. We conclude that the occurrence of the UUG initiation codon provides a mechanism for limiting the expression of *cya*, a gene essential for synthesis of the crucial cellular regulator cAMP.

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